Liquid biopsy: Comprehensive overview of circulating tumor DNA (Review)

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Abstract. Traditional tumor diagnosis methods rely on tissue biopsy, which can be invasive and unsuitable for long-term monitoring of tumor dynamics. The advent of liquid biopsy has notably improved the overall management of patients with cancer. Liquid biopsy techniques primarily involve detection of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). The present review focuses on ctDNA because of its significance in tumor diagnosis, monitoring and treatment. The use of ctDNA-based liquid biopsy offers several advantages, including non-invasive or minimally invasive collection methods, the ability to conduct repeated assessment and comprehensive insights into tumor biology. It serves crucial roles in disease management by facilitating screening of high-risk patients, dynamically monitoring therapeutic responses and diagnosis. Furthermore, ctDNA can be used to demonstrate pseudo-progression, monitor postoperative tumor status and guide adaptive treatment plans. The present study provides a comprehensive review of ctDNA, exploring its origins, metabolism, detection methods, clinical role and the current challenges associated with its application.

Contents

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1. Introduction

Currently, pathological tissue biopsy is the gold standard for diagnosing and monitoring a number of malignant tumors, such as lung cancer, stomach cancer, colorectal cancer. This method allows rapid assessment of the extent and nature of lesions, provides relatively accurate pathological classification and facilitates early detection and diagnosis. In addition, tissue biopsies conducted during treatment can reflect disease progression and treatment efficacy and provide clinicians with valuable information to tailor treatment plans (1). However, use of insufficient or inadequate tissue samples may lead to diagnostic bias, which may be exacerbated by tumor heterogeneity (2). Furthermore, repeated invasive procedures can cause discomfort for patients, particularly as the disease advances.

The emergence of liquid biopsy has effectively addressed several of these challenges. Liquid biopsy involves the molecular analysis of liquid (non-tissue) samples to evaluate physiological states (3). While blood samples are the most commonly used, other bodily fluids such as cerebrospinal fluid (CSF), saliva, pleural effusion, bile, abdominal fluid and urine can also be utilized (4). Liquid biopsy primarily focuses on analyzing circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), as well as circulating cell-free (cf)RNA, extracellular vesicles and tumor-inducing platelets (5). ctDNA has emerged as a pivotal element in clinical practice as it serves a key role in cancer diagnosis, monitoring and treatment. In clinical treatment, physicians are able manage treatment strategies by reference to ctDNA results. The detection of ctDNA pre-treatment and pre- and post-operation guide patient treatment plans (6). However, there are limitations to ctDNA analysis in liquid biopsy, including challenges in evaluating tumor pathology, detection sensitivity and the absence of standardized protocols.

2. Overview of ctDNA-based liquid biopsy

cfDNA. Circulating cfDNA refers to highly fragmented DNA released from cells into the bloodstream that circulates freely

in human blood (7). First discovered in 1948 (7), cfDNA has become a major focus of medical research (8-10). It is composed of both double- and single-stranded fragments (11-13), typically 120-220 bp in length, with an average length of ~167 bp, which is associated with the nucleosome (14).

In the bloodstream, cfDNA exists in three primary forms: Free, bound to protein (such as nucleosomes and lipoproteins) or associated with extracellular vesicles, such as exosomes, apoptotic bodies and microvesicles (15,16). The majority of plasma cfDNA is found in exosomes (17). The sources of cfDNA have been a topic of debate and can be generally divided into two main categories: Cellular destruction and active cellular release (18,19). Potential sources of cfDNA include cellular byproducts released during normal physiological processes, exogenous DNA originating from dietary intake, blood transfusions or infections, release from the nervous system, secretion into the blood circulation due to factors such as stress, hereditary conditions, degeneration or disease, fetal cellular material transferred to the mother during pregnancy and systemic release due to obesity and aging (19). Plasma cfDNA from healthy individuals predominantly originates from white blood cells (55%), red blood cell progenitors (30%) and vascular endothelial (10%) and liver cells (1%) (19). In patients with cancer, however, cfDNA is mainly derived from tumor tissue and surrounding cells (18).

cfDNA is a key biomarker for various physiological and pathological conditions and is associated with factors such as aging (20) and physical or psychological stress (21). In addition, cfDNA serves as a key biomarker in several types of cancer, including non-small cell lung cancer (NSCLC) (22), liver (23), breast (24,25), pancreatic (26), oral (27) and colorectal cancer (28,29). Previous studies suggest that cfDNA may also be associated with xenotransplantation (30,31).

cfDNA encompasses short and long DNA fragments and analysis of these fragments can provide valuable insight into a health status. Increasing evidence shows that cfDNA is vital for immune regulation, tumor-associated inflammation and the maintenance of cell homeostasis (32,33). cfDNA can impact cellular function and transformation, contribute to tumor growth and metastasis and holds promise for early disease detection (32,33). In healthy individuals, there is a dynamic balance between production and clearance of cfDNA. cfDNA is primarily cleared by the liver, spleen and kidney (34,35). However, in patients with chronic inflammation or tumors, cfDNA levels significantly increase due to impaired clearance and subsequent accumulation (32).

ctDNA. ctDNA is a subset of cfDNA that consists of DNA fragments that originate from tumor tissue and potentially other sources, such as shedding cells from normal tissue, characterized by genetic alterations that mirror those of the tumor (18,36). The increase in cfDNA levels in patients with cancer is primarily attributable to the elevated levels of ctDNA (37). Typically, ctDNA fragments are ~140 bp in length and have a half-life >2 h, which makes their dynamics variable (38,39). The detection rate of ctDNA in plasma ranging from 0.01% to a majority in the cfDNA, which reflects notable heterogeneity among different types of tumor (40,41). Despite this variability, the quantity of ctDNA detected is usually high compared with that of CTCs (42). The ability to detect ctDNA is influenced by characteristics of the tumor. For example, smaller solid tumors or those with low metabolic activity may be more challenging to detect, which may lead to false-negative results (6). Notably, ctDNA levels decrease rapidly following radical tumor resection if there is no or minimal residual tumor (43).

3. ctDNA detection in biological samples: Blood vs. non-blood sources

Blood. Blood is the most widely used sample type for detection of ctDNA (44), primarily due to the minimally invasive collection process and reproducibility. However, the precise mechanisms by which ctDNA enters the bloodstream remain unclear. It is has been hypothesized that ctDNA in blood originates from three primary sources: Apoptotic or necrotic and viable tumor cells and CTCs (45). Plasma is considered the optimal sample for ctDNA analysis, as serum (excluding clotting factors) contains an increased proportion of DNA from leukocyte lysis (46). For example, Heger *et al* (47) developed a molecular prognostic index for central nervous system (CNS) lymphoma using plasma ctDNA and demonstrated that is was effective in predicting patient outcomes. This ultra-sensitive method can detect CNSL-derived mutations in plasma ctDNA that are highly consistent with CSF and tumor tissue. Plasma ctDNA undetectable at baseline was associated with favorable outcomes. However, detecting ctDNA in white blood cells poses challenges due to high levels of cfDNA in these cells, which leads to notable dilution of the ctDNA (48). Compared with blood samples, ctDNA is easier to detect in non-blood samples.

CSF. Acquiring brain tissue for diagnostic purposes is both challenging and high-risk due to the unique structure and function of the brain. Therefore, CSF serves an irreplaceable role. While magnetic resonance imaging (MRI) is commonly used to monitor CNS diseases (49), its predictive capability is limited. Liquid biopsy that involves the detection of ctDNA in CSF may serve as a novel detection method for CNS diseases. The unique composition of CSF, along with the protective blood-brain barrier, decreases interference from cfDNA, which results in increased concentration of ctDNA and mutated allele frequencies (MAF) in CSF. This improves the sensitivity and accuracy of ctDNA mutation detection. Consequently, CSF biopsy may be a promising diagnostic tool for the detection and monitoring of brain tumors and CNS metastases (50-52).

A study has shown that carcinoembryonic antigen and CSF ctDNA are effective biomarkers for distinguishing patients with and without brain parenchymal tumor or CNS metastases (53). CSF ctDNA analysis has demonstrated distinct mutational profiles in patients with bone marrow metastasis (53). In a 2022 case report (54), a patient with lung adenocarcinoma experienced neurological symptoms, including headache, nausea, aphasia, limb restlessness and sudden blindness during treatment. While initial MRI and CSF cytology did not indicate the presence of brain metastasis, CSF ctDNA analysis identified the same *EGFR* mutation as that detected in the lung tumor of the patient. Follow-up MRI scans 9 months later confirmed brain metastasis, which suggested earlier MRI and CSF cytology results were false negatives.

This case underscores the potential of CSF ctDNA as an early diagnostic biomarker as it may detect brain metastasis before cytological or MRI evidence emerges.

To the best of our knowledge, studies of ctDNA in CSF have predominantly focused on adults (55-58), with relatively few investigating its application in children (59-61). Pages *et al* (62) assessed tumor reliability by analyzing ctDNA in peripheral blood, CSF and urine samples from children with brain tumors; ctDNA detection in this demographic was limited by low tumor fraction (TF). In numerous cases, the actual TF was >1%, with TF >0.1% being undetectable. Furthermore, only a small percentage of high-grade tumors were available. Therefore, uncertainty persists regarding the application of liquid biopsy for pediatric tumors, indicating the need for further research.

Saliva and sputum. The clinical relevance of saliva as a diagnostic tool is uncertain. A study from 2019 (63) suggested that saliva may not serve as an adequate alternative sample for quantitative cfDNA testing due to insufficient cfDNA concentration for diagnosis of NSCLC. However, it was proposed that saliva may be beneficial as a complementary method to cytology. Wang *et al* (64) made notable advancements by optimizing the extraction technique for sputum samples to overcome limitations posed by large amounts of mucus components and the low yield of cancer cells. Super-amplification refractory mutation system was used to analyze *EGFR* mutation status in cfDNA derived from sputum samples; sputum could be a promising sample type for detecting *EGFR* mutations, although its use for diagnosing lung adenocarcinoma may be limited. Further research by Wang *et al* (65) highlighted saliva from patients with lung adenocarcinoma as a valuable alternative source for detecting the *EGFR* exon 20 p.T790M mutation, which is linked to resistance to EGFR targeted therapy (65). In addition, a previous study investigating head and neck cancer reported a high concordance (93%) in ctDNA detection between saliva and blood samples, as well as efficacy of ctDNA in saliva in predicting patient outcomes (66). A meta-analysis of 64 cases of malignant salivary gland carcinoma found increased levels of ctDNA and CTCs in malignant cases (67). According to the 2024 Expert Consensus, ctDNA extracted from saliva, along with serum or plasma, provides meaningful insight into tumor genetics and dynamics (68).

Pleural, peritoneal and pericardial effusion. Tumor supernatant, such as pleural, peritoneal and pericardial effusion, are in proximity to tumors and may provide distinct advantages over blood for ctDNA detection; for example, it is easier to detect ctDNA of abdominal tumor with abdominal fluid A previous study that compared mutant allele scores from 30 supernatant samples with those from paired formalin-fixed paraffin-embedded cell blocks reported a variant concordance up to 90% (69), and similar results were detected in both supernatant and FFPE samples in 74% of cases. This suggests that supernatant may serve as a viable alternative to traditional tissue biopsy (69).

Yang *et al* (70) used high-throughput next generation sequencing (NGS) to analyze cfDNA in 15 pleural, five abdominal and one pericardial effusion; they identified key pathogenic mutations in malignant fluid from 13 patients with metastatic tumors, potentially malignant fluid from two cases and benign fluid from one case. In another study that focused on peritoneal cancer, malignant ascites or peritoneal lavage fluid was collected for microdroplet digital PCR (ddPCR) analysis; peritoneal effusion cfDNA could predict the tumor load of peritoneal cancer and assess patient eligibility for cytoreductive surgery by calculating the MAF (71). Compared with blood, fluids such as pleural and pericardial effusion and ascites exhibit increased sensitivity for ctDNA testing and may serve as predictive biomarkers for responses to EGFR inhibitors (48).

Pancreatic fluid and bile. In pancreaticobiliary tract tumors, obtaining tissue biopsies can be challenging due to the occult nature of the disease. Given their direct contact with tumor tissue, bile and pancreatic fluid are ideal samples for liquid biopsies. Kinugasa *et al* (72) compared levels of ctDNA in tumor tissue with those in bile from 49 patients with gallbladder cancer; ctDNA isolation from bile was a valuable approach for diagnosing gallbladder cancer. The sensitivity of ctDNA testing (58.3%) was higher compared with that of cytology (45.8%), and there was a high mutation concordance between the two methods. Further study has demonstrated consistent *KRAS* mutations in ctDNA from bile, plasma and formalin-fixed paraffin-embedded samples from patients with bile duct tumors (39). Notably, only 18.8% of plasma ctDNA samples test positive for *KRAS* mutations, compared with a detection rate of 48.0% in bile ctDNA (39). Moreover, patients with *KRAS* mutations detected in bile ctDNA exhibit significantly lower survival rates compared with those with wild-type *KRAS* (39).

In early-stage pancreatic ductal adenocarcinoma, identification of mutations in plasma ctDNA is often challenging (73). A study in 2023 compared the detection rates of ctDNA sourced from pancreatic fluid with that in plasma; DNA concentrations and the ratios of Alu247/Alu115 were higher in pancreatic fluid compared with plasma, however, there was no difference in the mutation detection rate between pancreas and plasma (74). This limitation may be attributable to the small sample size and influence of enzymes present in pancreatic fluid, which underscores the need for further investigation.

Urine. In 1995, ctDNA was detected in urine (75), marking the beginning of research interest in this non-invasive biomarker. Thus far, two primary sources of ctDNA in urine have been identified, including debris shed directly from tumor cells within the urinary system and CTCs that are filtered through the kidney (76,77). The latter source of ctDNA tends to have a smaller molecular weight, restricted by pore size of the glomerular barrier (76). In 2008, a comparative study examined *KRAS* mutations in both blood and urine samples from patients with colon cancer; Although the study had a small sample size, mutation rates were similar in both fluids. However, as the sample size increased, mutation rates in urine became significantly higher compared with those in blood (78), highlighting potential of urine as a tumor marker.

A recent prospective multi-center study reported that measurement of DNA methylation in urine effectively differentiates pathological types of bladder cancer and predicts 180-day recurrence-free survival with 100% accuracy (79).

This represents a breakthrough in use of urine DNA methylation for differentiation of pathological cancer types. In addition, a study on ctDNA in urine during neoadjuvant chemotherapy for bladder cancer demonstrated that monitoring tumor DNA dynamics in urine, supernatant and plasma predicts treatment response and outcome (80). Urinary ctDNA has also been shown to detect the recurrence of upper urinary tract urothelial carcinoma up to 60 days earlier than cystoscopy (81). Kim *et al* (82) reported that binding urinary ctDNA improves detection rate of hepatocellular carcinoma from 62 to 92% (82). Increased DNA methylation levels in urine have also been reported in patients with NSCLC.

Urine sampling is a non-invasive procedure that can be performed by non-professionals. Patients can conveniently collect samples at home, which can be sent to testing laboratories. Unlike blood, which is subject to buffering and regulatory mechanisms that may alter its properties (83), urine is excreted and may better reflect bodily abnormality. In addition, urine has a lower presence of contaminating proteins compared with blood, which simplifies DNA extraction processes (76). The ability to test large volumes of urine repeatedly enhances sensitivity of ctDNA detection (84), which makes it a promising tool in cancer diagnostics.

Semen. To the best of our knowledge, prostate-specific antigen is the only well-established tumor marker for prostate cancer. However, its specificity is limited, necessitating exploration of additional diagnostic tools. ctDNA testing has emerged as a valuable adjunct in diagnosis of prostate cancer (85-87). While ctDNA is commonly detected in advanced cancer such as pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, hepatocellular and head and neck cancer and melanoma, it is present in <50% of cases of primary brain, kidney, prostate and thyroid cancer (40).

Semen may serve as a potential sample for prostate cancer diagnostics. Significant differences in cfDNA levels have been observed (83) in semen samples from patients with prostate cancer, individuals with benign prostatic hyperplasia and healthy individuals (88). Patients with prostate cancer exhibit higher concentrations of cfDNA than healthy individuals in semen alongside a distinctive size distribution of cfDNA fragments. Notably, longer cfDNA fragments are significantly more prevalent in semen from patients with prostate cancer compared with those with benign hyperplasia or healthy individuals (69). Size and concentration of cfDNA fragments in semen is associated with tumor burden and treatment response (88). As a direct source of prostate disease-specific molecules, semen represents a promising body fluid for identification of prostate cancer biomarkers and may provide important insight for early diagnosis (Fig. 1) (89).

4. Detection methods

ctDNA provides insight for cancer diagnosis and treatment. To harness the potential of ctDNA, accurate detection methods are key. The main techniques for ctDNA detection include DNA sequencing, PCR-based methods and DNA-based hybridization strategies (90-92). At present there is no universally accepted standard of detection (93).

DNA sequencing

Directed error correction sequencing. Phallen *et al* (94) developed targeted error correction sequencing (TEC-Seq), which enables ultra-sensitive evaluation of sequence changes in ctDNA through large-scale parallel sequencing. TEC-Seq uses targeted capture of multiple genomic regions combined with deep sequencing of DNA fragments, facilitating the detection of 58 cancer-associated genes spanning 81 kb. A plasma analysis conducted on 44 healthy individuals reported that 16% of asymptomatic individuals exhibit genomic changes associated with clonal hematopoiesis, although none show alterations in driver genes linked to solid tumors (94). CancerSEEK assay (multicancer early detection blood test) demonstrates an accurate tissue-of-origin prediction in 83% of cases, while TEC-Seq yields cancer detection rates of 59-71%, depending on the type of cancer assessed (95). Achieving effective sensitivity in ctDNA analysis using TEC-Seq presents a notable technical challenge (94), which reflects the need for continued refinement of this approach to enhance its applicability in clinical settings.

NGS. Since US Food and Drug Administration (FDA) approval of the Guardant360® CDx (Guardant Health, Inc.) for use of third-generation tyrosine kinase inhibitor osimertinib in patients with NSCLC and *EGFR* mutations, the NGS approach has rapidly evolved (96). NGS is the preferred liquid biopsy technique for metastatic NSCLC according to the current European Society for Medical Oncology guidelines (97). This high-throughput sequencing method enables comprehensive analysis of DNA and RNA, which allows examination of the entire sequence of target genes and facilitates detection of a broader spectrum of mutations, including previously unknown variants (97,98). NGS can simultaneously sequence multiple genomes on a single platform, even when working with low concentrations of tumor DNA derived from plasma or other liquid biopsy samples (99). NGS can be tailored to analyze a variable number of regions, from a few loci to the entire exome or genome (100). Broadly, NGS targeting ctDNA uses two main approaches. The first approach involves deep sequencing of specific regions containing relevant mutations, which offers high sensitivity and specificity suitable for targeted clinical application. The second approach entails whole-exome or whole-genome sequencing, which can uncover novel genomic changes and is more suitable to basic research (98). NGS is characterized by high throughput, sensitivity and specificity, rendering NGS-based ctDNA mutation spectrum analysis superior to other technologies (98) such as TEC-Seq. However, there are challenges associated with NGS, including the potential for mislocalisation of mutations (97). In addition, this technology requires extensive data analysis, incurs notable costs and has a turnaround time of 7-14 days (101,102).

Targeted amplicon sequencing (TAm-Seq). TAm-Seq is a labeled amplicon deep sequencing method that integrates efficient library preparation with advanced statistical analysis. This technique enables the sequencing of ~6,000 nucleotides and in-depth analysis (103). A notable implementation of TAm-Seq is InVisionFirst® (Neogenomics Laboratories).

Liquid biopsy platform, which is designed to detect both hotspot mutations and entire coding regions across 35 cancer-associated genes. Leveraging enhanced TAm-Seq

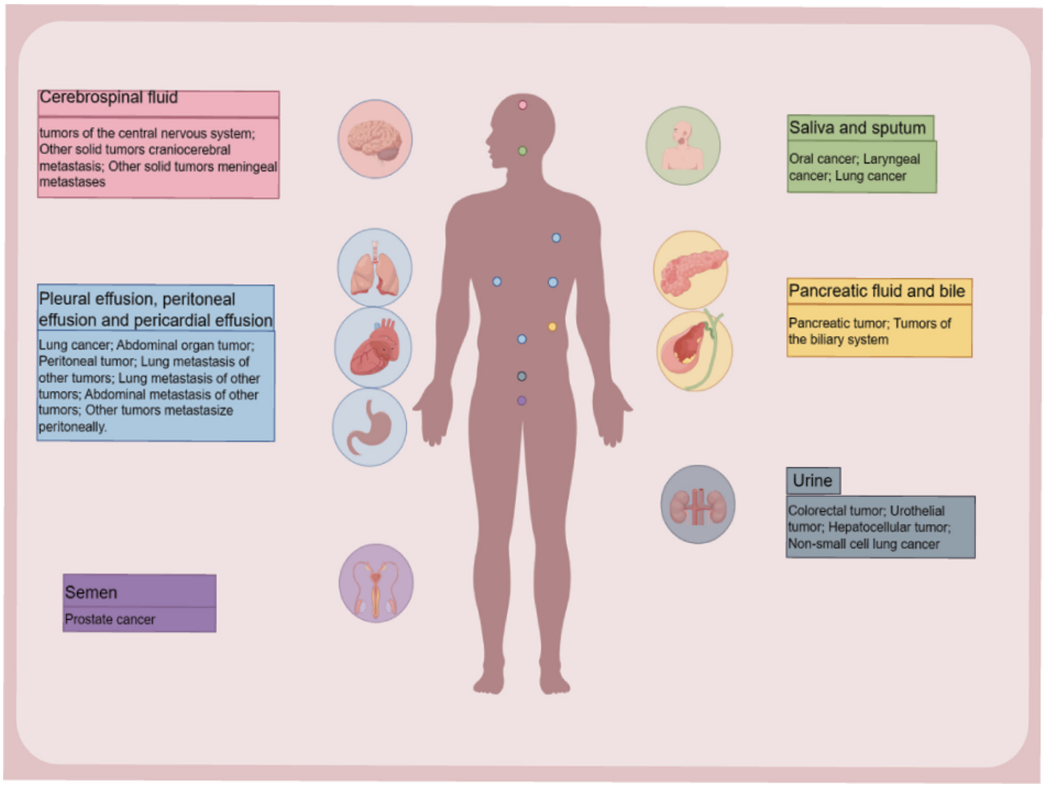

Figure 1. ctDNA samples. In addition to blood samples, ctDNA can be detected in pleural, peritoneal and pericardial effusion, cerebrospinal fluid, saliva and sputum, pancreatic fluid and bile, urine and semen. ct, circulating tumor.

techniques, it identifies low-frequency mutations in ctDNA by amplifying highly fragmented DNA (104). Further improvements of Tm-Seq involves optimization of the amplification process by splitting it into two steps. The initial step involves limited cycle pre-amplification using all primer sets to capture the starting molecules present in the template. This is followed by a single amplification step to purify and isolate the target sequence. This refined approach enables detection of cancer mutations in ctDNA at allelic frequencies as low as 2% with sensitivity and specificity of >97% (105). TAm-Seq method demonstrates its utility in clinical settings by routinely detecting ctDNA not only at the time of diagnosis, but also post-treatment (106).

Deep sequencing personalized cancer analysis (CAPP-Seq). Developed by Newman *et al* (107), CAPP-Seq is an economical and ultra-sensitive method for quantifying ctDNA. This method integrates a low DNA initiation mass library preparation strategy with a multiphase bioinformatics approach to generate a 'selector' of biotinylated DNA oligonucleotides designed to target tumor regions with recurrent mutations. To monitor ctDNA, the selector is initially applied to tumor DNA to identify unique cancer-specific genetic aberrations. Subsequently, it is used on ctDNA to quantify these aberrations (107). Among the various NGS-based ctDNA analysis methods, CAPP-Seq has the lowest background error rate and detection limit (107), demonstrating greater sensitivity compared with TAm-Seq (106). Originally intended for analysis of NSCLC (107), CAPP-Seq has since been successfully applied to a variety of other types of cancer, including esophageal (108) and ovarian cancer (109), mantle cell lymphoma (110), bladder cancer (111), head and neck squamous cell carcinoma (112) and melanoma (113). However, despite its high sensitivity, CAPP-Seq has higher cost compared with TAm-Seq and may not offer advantages for routine screening and surveillance (106).

PCR

Real-time quantitative PCR (qPCR) qPCR can be used for endometrial cancer (114), non-small cell lung cancer (115), colorectal cancer (116). qPCR-based tests have received approval from US FDA and European Medicines Agency for detection, activation or identification of resistance to EGFR targeted therapies in NSCLC (117). Despite its widespread use, the simple nature of these tests may increase the risk of false positives when qPCR is used in isolation (97).

Microdroplet ddRCR. Microdroplet ddPCR, also known as third-generation PCR, uses sample allocation, restricted dilution and statistical data processing based on Poisson distributions to accurately and reliably quantify nucleic acids. This technique divides mixed nucleic acid molecules and PCR solution into small droplets. By using microfluidic loops and surfactant chemistry, sample DNA is randomly assigned to isolated droplets, generating 20,000 droplets. The template is amplified and product is detected based on specific fluorescent labeling (97,118,119). ddPCR is well-suited for studying specific single-gene hotspot mutations that may be found in CSF samples, achieving a limit of detection as low as 0.01%/reaction (119,120). It can measure mutations that

constitute 0.01% of a sample (39), offering greater sensitivity compared with qPCR (97). However, compared with NGS, ddPCR has narrower reference range (90.0% of operable mutations) and does not cover certain variants, such as estrogen receptor 1 mutation (121). In addition, ddPCR requires specialized personnel to operate, which adds to overall complexity and operational costs (39,118,122).

Beads, emulsion, amplification, magnetics (BEAMing). BEAMing is a digital PCR method that enhances the capability of ddPCR by incorporating pre-amplifications of DNA using conventional PCR and target-specific primers (97). PCR products amplified by BEAMing molecules are linked to single magnetic beads and the mutation sites extended via fluorescent probes or primers. By counting fluorescently labelled beads, BEAMing allows the quantitative detection of mutant alleles (119,123). Taniguchi *et al* (123) used BEAMing to monitor disease progression in patients with lung cancer undergoing EGFR targeted therapy, which effectively determined the proportion of T790M-positive alleles in cancer cells, regardless of potential contamination from normal cell DNA.

Thermal coupling index expansion. An ultra-fast monitoring method known as thermal coupling exponential amplification test has been recently reported (124). This technique combines exponential amplification reaction (EXPAR) with *Thermus thermophilus* argonaute-coupling), from the thermophilic bacterium *Thermus thermophilus*, to quickly and accurately detect ctDNA in ~16 min (124). A previous study on tumor threshold changes in mouse models (seven Kirsten rat sarcoma-2 virus (KRAS) point mutations) indicated that this method holds significant potential for monitoring tumor load and evaluating chemotherapy response (121). TtAgo-CEAR assay leverages rapid, specific cleavage function of TtAgo and the high amplification efficiency of EXPAR to identify common hotspot mutations in *KRAS* (124).

DNA hybridization. Traditional methods for detecting and quantifying ctDNA, such as PCR and NGS, are well-established but have limitations (125-127). These methods are not suitable for detecting short ctDNA fragments (<100 bp). Moreover, they can be costly, require complex instrumentation, time-consuming due to multiple reaction steps and prone to false positives (128). By contrast, hybrid chain reaction is an isothermal, enzyme-free amplification technique that allows indefinite amplification of signals. This method provides advantages for the detection of small molecules and shows potential for ctDNA detection (129). In 2021, researchers successfully employed a hydrogel-based hybrid chain reaction to amplify small amounts of exosomal microRNA from urine samples, achieving a 35-fold increase in detection sensitivity and effectively distinguishing patients with prostate cancer from normal controls (130). A novel device known as the hybrid chain reactor-driven laboratory fiber optic device has been introduced for ultra-fast and sensitive detection of ctDNA in whole blood. This method is time-efficient, straightforward and cost-effective, as it enables real-time monitoring of ctDNA changes (128) and represents a promising direction for advancing detection capability.

5. Clinical role of ctDNA

Screening and management of patients with cancer. ctDNA serves a key role in the screening and early diagnosis of various solid types of tumors, particularly among asymptomatic individuals. Phallen *et al* (94) demonstrated a strong correlation between plasma somatic mutations and tumor changes in patients with stage I or II colorectal, ovarian and breast cancer. This suggested that ctDNA analysis may be instrumental in both early detection and ongoing disease management. In a study of esophageal adenocarcinoma, baseline ctDNA levels were used to identify patients with locally advanced disease at higher risk of relapse (106). This highlights potential of ctDNA not only as a biomarker for early diagnosis but also as a prognostic tool for tailoring treatment and monitoring disease progression.

Dynamic longitudinal monitoring to evaluate prognosis. The role of ctDNA as a prognostic marker has gained recognition in recent years (131-133). ctDNA is detected in various solid tumors, with its concentration associated with the stage of the disease (134). In a prospective phase II clinical trial, ctDNA was dynamically monitored every three treatment cycles in five patients with solid tumors undergoing immune checkpoint inhibitor (ICI) treatment. The ctDNA levels were associated with tumor status, demonstrating predictive value both at baseline and following treatment (135). The presence of ctDNA following surgery is strongly indicative of tumor recurrence (6). Gale *et al* (136) demonstrated that ctDNA could identify residual lesions and predict recurrence in patients with NSCLC. Pre- and post-treatment ctDNA testing is shown to identify patients with NSCLC at high risk for recurrence (136). Monitoring ctDNA levels at baseline, during neoadjuvant and adjuvant therapy and after radical therapy allows clinicians to assess drug response and refine treatment regimens. This dynamic longitudinal monitoring ultimately improves prognostic evaluation and informs clinical decision-making, contributing to improved patient management and outcome.

Effectively identifying false advances. Response Evaluation Criteria in Solid Tumors (RECIST1.1) guidelines are key for assessing tumor progression (137). However, they also have limitations, particularly concerning pseudo-progression, which refers to the transient appearance of space-occupying lesions and edema following treatment. This can often mimic disease progression on radiographical imaging but typically resolves or changes after 4-8 weeks of follow-up (138). In clinical practice, clinicians rely on RECIST guidelines to evaluate disease progression and make treatment decisions. However, ctDNA detection can offer earlier and more accurate indication of disease status compared with imaging, potentially reducing follow-up time and offering better guidance for clinical treatment. Similarly, the emerging concept of 'hyperprogression' describes a rapid acceleration in tumor growth that can be induced by ICIs (139). To the best of our knowledge, no studies have reported the association between ctDNA and hyperprogressive disease. Future research should explore this association and its implications.

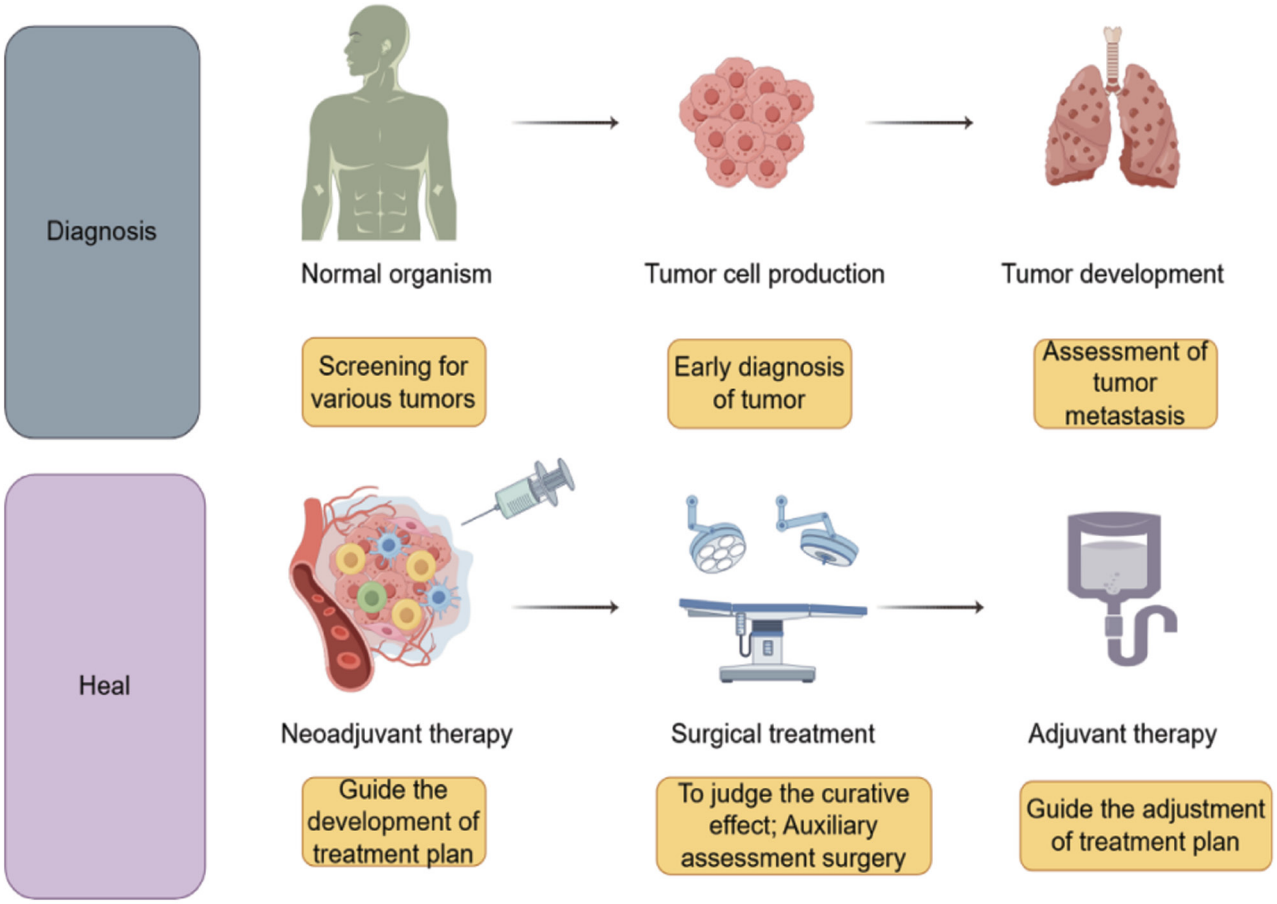

Figure 2. ctDNA in patients with cancer. ctDNA detection may facilitate diagnosis and treatment strategies. ct, circulating tumor.

Detecting molecular residual disease (MRD). Detection of ctDNA provides extensive information and enables analysis of minimal residual lesions. Early detection of residual ctDNA following local radical treatment can indicate MRD and identify patients at higher risk of recurrence or metastasis (140). Due to limited sensitivity of CTC detection, it is rarely used for MRD evaluation (141). Over the past two decades, early detection of MRD in children with acute lymphoblastic leukemia has significantly improved risk stratification, enhanced treatment for high-risk patients and decreased treatment intensity for those at low risk (142). The potential for MRD detection is established in other malignancies, including acute myeloid (143,144) and chronic lymphocytic leukemia (145), NSCLC (146,147), multiple myeloma (148-151), breast cancer (152), melanoma (153), head and neck squamous cell carcinoma (154), follicular lymphoma (155), urothelial carcinoma (156) and colorectal cancer (157).

Guiding treatment escalation and de-escalation. A promising application of ctDNA is its ability to inform decisions regarding treatment escalation and de-escalation (158). Patients with a positive MRD result may be candidates for intensified adjuvant therapy, while those with a negative MRD result may potentially benefit from a reduction in treatment intensity (43). It has been suggested that a key treatment endpoint for colorectal cancer should be complete clearance of ctDNA (43). Currently, a multicenter, prospective, randomized clinical trial is underway to evaluate efficacy of ctDNA-guided adjuvant chemotherapy strategies compared to standard care (158). This aims to assess whether ctDNA-guided treatment adjustments yield superior outcomes in terms of three-year disease-free survival for patients with high-risk stage II and III colorectal cancer (Fig. 2) (158).

6. Limitations

A notable challenge associated with ctDNA-based liquid biopsy is limited detection capability. The mutation abundance of ctDNA is often lower compared with that in localized tumor tissues, and its detectability is influenced by factors including tumor type and load, anatomical location, cellular turnover and disease stage (141). In the context of early cancer detection, ctDNA levels are particularly low, often causing MAF to fall below detection limits of current methods (159). Thus, improving sensitivity of existing detection methods is key. Employing a combination of DNA analysis from liquid biopsy and tissue samples may improve the overall sensitivity and diagnostic accuracy (160).

Discrepancies in results can arise from the diverse standards and interpretations employed by different ctDNA testing methods and laboratories. There is need for the establishment of standardized testing protocols and interpretative guidelines. It is also important to select the appropriate sampling methods, as improper collection of cfDNA from body fluids can lead to missed detection of ctDNA even with appropriate testing methods (100).

Another barrier is the high cost associated with ctDNA detection technologies and equipment, which hampers comprehensive clinical monitoring and may affect treatment decisions. Furthermore, there is currently no evidence to suggest that ctDNA can fully replace traditional pathological testing. Addressing these challenges is key for refining the role of ctDNA in clinical practice.

7. Outlook

ctDNA may serve a key role tumor treatment. Despite existing challenges, ongoing research may advance the utility of ctDNA in clinical practice. As improvements in detection sensitivity, standardization of testing protocols and cost reduction are realized, ctDNA may enhance patient care by guiding treatment decisions, monitoring therapeutic response and improving outcomes.

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Authors' contributions

QG and ZYZ conceived the study. QG, ZYZ, SL, JM and ZZ wrote and reviewed the manuscript. Data authentication is not applicable. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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